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Single-Molecule Detection Technologies in Miniaturized High Throughput Screening: Binding Assays for G Protein–Coupled Receptors Using Fluorescence Intensity Distribution Analysis and Fluorescence Anisotropy

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ABSTRACT

G Protein–coupled receptors (GPCRs) represent one of the most important target classes for drug discovery. Various assay formats are currently applied to screen large compound libraries for agonists or antagonists. However, the development of nonradioactive, miniaturizable assays that are compatible with the requirements of ultra-high throughput screening (uHTS) has so far been slow. In this report we describe homogeneous fluorescence-based binding assays that are highly amenable to miniaturization. Fluorescence intensity distribution analysis (FIDA) is a single-molecule detection method that is sensitive to brightness changes of individual particles, such as those induced by binding of fluorescent ligands to membrane particles with multiple receptor sites. As a confocal detection technology, FIDA inherently allows reduction of the assay volume to the microliter range and below without any loss of signal. Binding and displacement experiments are demonstrated for various types of GPCRs, such as chemokine, peptide hormone, or small-molecule ligand receptors, demonstrating the broad applicability of this method. The results correlate quantitatively with radioligand binding data. We compare FIDA with fluorescence anisotropy (FA), which is based on changes of molecular rotation rates upon binding of fluorescent ligands to membranes. While FA requires a higher degree of binding, FIDA is sensitive down to lower levels of receptor expression. Both methods are, within these boundary conditions, applicable to uHTS.

INTRODUCTION

G PROTEIN–COUPLED RECEPTORS (GPCRs) sense extracellular signals such as hormones and biogenic amines and transmit the signal into the cell by interacting with heterotrimeric G proteins.[1] GPCRs are involved in a wide range of signaling pathways, and play a role in a variety of disease states. In addition, the ligand binding sites of GPCRs are exposed extracellularly and therefore readily available for interaction with drugs (c.f. intracellular targets such as kinases). These characteristics make GPCRs one of the most important classes of therapeutic targets for drug discovery, as proven by the historical success of drugs that modulate receptor activity. Moreover, genomic approaches have allowed the identification of many new members of this class (so-called orphan receptors), which are providing a rich source of new targets for potential therapeutic intervention. Hence, major screening efforts are being undertaken to identify ligands for such receptors and to search for novel antagonists or agonists.[2]

However, GPCRs present considerable challenges for screening, particularly with respect to the overall goals of miniaturizing and simplifying assay formats and increasing throughput. High throughput screening (HTS)–compatible functional assays are required for ligand identification (so-called ligand fishing[2]) or agonist screening, and in this area considerable advances have been made, particularly using measurements of intracellular Ca^{2+} mobilization[3,4] or reporter gene technology.[5] In contrast, binding assays are generally preferred for antagonist-directed HTS or for structure–activity relationship (SAR) screening of drug candidates, because this approach produces more quantitative information on the thermodynamics of compound binding. However, in this case, expression of sufficient level of receptor to allow the development of robust binding assays can be difficult.

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As integral membrane proteins, GPCRs can only be purified after solubilizing the membrane, and biological assays normally require reconstitution of a lipid-bound form. Because expression, purification, and reconstitution of reagent quantities of receptors in a reproducible manner is difficult, membrane fractions with a receptor density of typically less than 0.1% of total protein are usually employed for ligand binding assays. While homogeneous fluorescence-based miniaturized assay methods are well established for soluble targets,[6–8] they are more difficult to apply to this type of membrane preparation because of the relatively low abundance of the primary target and significant background from, for example, light scattering. The most common approaches currently used for GPCR binding assays employ radiolabeled ligands and either separation-based (i.e., filter binding) or homogeneous counting methods.[9] These assays perform adequately in 96- or 384-well plate formats but are very difficult to miniaturize further. Recently, a homogeneous fluorescence-based approach using laser-scanning imaging to detect ligand binding to whole cells has been used in 96- and 384-well plate formats.[10,11] In addition, the use of fluorescence anisotropy has recently been reported for measurement of binding of fluoro-labeled ligands to a range of GPCRs, albeit not below a total volume of 40 μl in 384-well format.[12]

Clearly, progress in miniaturizing assays for these targets to formats compatible with uHTS (our working definition of uHTS, in agreement with the general usage of this term, is $\geq 10^5$ assays performed per 24 h in high-density plates [e.g., 1536-well or low-volume 384-well plates] and using assay volumes of ≤ 10 μl) has thus far been slow. In this paper, we demonstrate miniaturized homogeneous fluorescence-based GPCR ligand binding assays based on fluorescence intensity distribution analysis (FIDA) in comparison with the more conventional macroscopic fluorescence technique of fluorescence anisotropy (FA). The resultant assays are highly amenable to miniaturization (FIDA down to 1-μl and FA to 10-μl assay volumes) and well suited to uHTS.

FIDA has recently been developed as a confocal detection method that allows the determination of concentrations and specific brightness values of individual fluorescent species in a mixture.[13,14] Similar to fluorescence correlation spectroscopy (FCS), FIDA is based on the detection of temporal fluctuations of emitted light from a small confocal volume ($\sim 10^{-15}$ L) that, at nanomolar tracer concentrations, contains on average only a few fluorescent particles. Fluorophores passing through the detection volume cause bursts of fluorescence. The duration of these bursts reflects the diffusion time (and therefore the mass) of the fluorescent particles, while the amplitude of the photon bursts contains information about the brightness of individual particles. Analysis of the time-dependent behavior of the fluorescence fluctuations by calculating autocorrelation functions allows analytical determination of the diffusion characteristics and concentration of fluorescent particles, which is the basis for mass-dependent FCS assays.[6–8,15] In comparison, analysis of the amplitude information in the fluctuation signal can be used to determine the molecular brightness and concentration of fluorescent particles for mass-independent but brightness-dependent FIDA assays. FCS and FIDA therefore use the same primary data (fluorescence intensity fluctuations) but provide complementary information about the sample analytes. Both FIDA and FCS are intrinsically highly amenable to miniaturization because the signal output is independent of the total assay volume.[6,7]

FIDA can be used to configure biochemical assays if the fluorescent analyte undergoes a change of brightness. The brightness of particles independently diffusing through the detection volume can be affected in two ways: (1) an individual fluorophore may be subject to chemical reactions, quenching, or other environmental effects influencing its specific brightness; or (2) the number of fluorophores bound to a single particle may change. An example wherein both effects are observed has been discussed by Kaask et al.[13] Examples of the latter type of brightness changes are beads or membrane vesicles with multiple receptor sites that allow accumulation of multiple fluorescent ligands binding to a single diffusing particle.[25] As an application of FIDA based on this principle, we describe herein the configuration of homogeneous FIDA binding assays for GPCRs. (Bead-based applications will be described in a subsequent report.) FA can also be used to detect the binding of low-molecular-weight fluoro-ligands to membrane bound receptors based upon differences in fluorophore molecular rotation rates.[6] In this paper, we compare results obtained from both techniques used to measure binding of fluorescent-labeled ligands to a range of GPCRs.

MATERIALS AND METHODS

Fluoro-ligands and receptors

BODIPY® FL-CGP12177 was purchased from Molecular Probes (Leiden, The Netherlands), and Rhodamine Green-GRP was custom-synthesized by Research Genetics (Huntsville, AL). All other fluorescently labeled ligands were prepared in the Molecular Interactions & New Assay Technologies group at SmithKline Beecham Pharmaceuticals (Harlow, UK), using labeling reagents from Molecular Probes and reaction conditions as recommended by the manufacturer. Melanin-concentrating hormone (MCH) peptide was purchased from Bachem (Merseyside, UK); all other chemicals were from Sigma (Dorset, UK). Low-molecular-weight antagonists were synthesized at SmithKline Beecham. Membrane vesicles were prepared by lysing HEK293 or CHO cell lines stably expressing the respective receptor in hypotonic buffer in a Dounce homogenizer, and two subsequent centrifugation steps (10 min at $500g_{max}$ for removing unlysed cells and nuclei, 20 min at $100{,}000g_{max}$ to isolate membranes).

FIDA Measurements

FIDA measurements were performed on confocal microscope–based FCS/FIDA readers (either a modified ConfoCor [EVOTEC BioSystems, Hamburg, and Carl Zeiss, Jena, Germany] or an EVOTEC FCS$^+$ plus reader based upon an Olympus microscope body) using an argon ion laser (488 nm line) or a helium-neon laser (543 nm line) as excitation light sources. Read times were 10 s/well, unless stated otherwise. The instruments were equipped with rapid beam-scanning devices allowing movement of the detection volume within the sample well, to eliminate the dependence upon diffusion for sampling of vesicles/ligands. Typically, for a single read time of 10 s, an area of 0.02 mm^2 was scanned, with the laser beam focused to a spot of approximately 0.5 μm radius and the focus adjusted to a height

of 180 μm into the solution. The sampling time for photon counting was set to 40 μs. Because the algorithm for evaluating FIDA can tolerate only limited change of coordinates of fluorescent particles during the sampling time interval of 40 μs the scanning speed was adjusted to yield an apparent diffusion time of at least five counting intervals (200 μs total), which also is the typical diffusion time of tetramethylrhodamine (TAMRA) molecules in a stationary detection volume in the same optical setup as determined by FCS. For convenience, most measurements were performed on glass-bottomed 96-well plates (Whatman Polyfiltronics, Maidstone, UK), although, by the nature of the FIDA technique, equivalent data are obtained irrespective of assay volume. From the primary data (time-dependent fluorescence intensity trace), the distribution of number of photons detected per counting interval was calculated, and these photon counting distributions were analyzed by either inverse transformation with regularization (ITR) or multicomponent fitting (MCF) using FCS$^+$ plus software (EVOTEC BioSystems),[13,25] to yield the concentration of bound ligand and the fractional binding.

Fluorescence anisotropy measurements

FA measurements were performed on an Acquest™ plate reader (LJL BioSystems, Sunnyvale, CA) using black 1536-well plates (Greiner, UK) or low-volume 384-HE plates (LJL Biosystems, Surrey, UK). Filters used for measuring TAMRA labels were 530(25)/580(10)-nm bandpass filters for excitation/emission, and a dichroic filter with cutoff at 561 nm. All measurements were calculated as anisotropy (A) instead of the historically more widely used polarization (P) because in two- (or more) component systems the observed anisotropy is simply the sum of the anisotropies of individual components weighted by their brightness values, whereas for polarization the relation is more complicated.[6] Therefore, quantitative analysis of binding reactions is easier in terms of anisotropy. Anisotropy is calculated from perpendicular and parallel emission intensities according to $A = (I_{par} - I_{perp})/(I_{par} + 2I_{perp})$, and is related to polarization by $A = 2P/(3 - P)$.

RESULTS AND DISCUSSION

Ligand binding to membrane-bound receptors measured by FIDA and FA

When fluorescently labeled ligands bind to membrane vesicles containing GPCRs, very bright particles are formed as a result of the multiple stoichiometry of ligand binding per particle. In a confocal detection mode and at suitable concentrations, these particles cause large-amplitude bursts in the fluorescence intensity trace ($F_{(t)}$), which can be examined by histogram analysis of the frequency ($P_{(n)}$) with which a certain number of photons is detected per sampling time. These histograms have also been termed burst size distributions[16] or photon counting histograms.[17] Bright particles are reflected in $P_{(n)}$ by a distribution curve extending to high numbers of photons per sampling time. As an example, FIDA measurements on TAMRA-MCH binding to MCH receptor[18] membranes are shown in Fig. 1A. The control for nonspecific binding (NSB), which contained a 1,000-fold excess of unlabeled MCH, shows a much narrower distribution (Fig. 1A).

Quantitative evaluation of these signals requires a theoretical model to describe $P_{(n)}$, for which various approaches have been published.[13,16,17] The model used needs to account adequately for the nonhomogeneous distribution of light intensity ($B_{(r)}$) within the sample volume. In FIDA, this is done by combining spatial sections of equal B values and modeling the spatial brightness function $B_{(r)}$ as a one-dimensional relationship between brightness and volume elements, which then can be described by the first three terms of a polynomial representation. The three parameters needed to characterize this representation of $B_{(r)}$ are determined by calibration measurements on a single species, and here the unbound dye (TAMRA in this case) was used. The contribution of a single fluorescent species and a single set of volume elements with a specific B value to $P_{(n)}$ is then given by a double-poissonian distribution. The first poissonian describes the probability of finding a certain number of molecules within the volume element, and the second one describes the probability of detecting a certain number of photons per sampling time for a given number of molecules with a specific brightness. $P_{(n)}$ could then in principle be calculated by convolution of all contributions from different fluorescent species and different volume elements. However, in FIDA an alternative method has been realized that simplifies this calculation step; introducing the representation of generating functions allows the replacement of convolution integrals by simple spatial integrals, and $P_{(n)}$ may be calculated by adding all single contributions of different species.[13]

Using these tools to calculate $P_{(n)}$, two methods of data analysis have been employed in FIDA[13]: ITR[19] and MCF analysis. Using the ITR method, concentrations for a set of components with predefined brightness values are determined, employing boundary conditions such as nonnegative concentrations and a continuous distribution with finite halfwidth of peaks for single species. The ITR fitting procedure results in a brightness distribution curve generated by the calculated discrete concentration values (e.g., for 50 components in the range from 1 to 10,000 kHz, evenly distributed on a logarithmic scale, see Fig. 1B). The corresponding fitted curve agrees well with the measured $P_{(n)}$ (Fig. 1A). The brightness distribution curve obtained for the NSB sample shows only a single peak at low brightness representing the free nonbound ligand, whereas, for the total-binding curve, components in the high-brightness range are als found, indicating multiple ligands binding to membrane vesicles (Fig. 1B). Bound and unbound species can easily be discriminated according to their brightness, and can be quantified by summation of all concentration values in the range of free or vesicle-bound ligand, respectively, which in this example yields 29% binding.

In MCF analysis, in comparison, only a limited number of species (typically two to three) with distinct brightness values are assumed to represent the brightness distribution in the sample. As an example, the photon counting histogram obtained for total binding was analysed by two components reflecting free ligand and ligand-stained vesicles, respectively. The fit curve obtained assuming two components is similar to the ITR fit (Fig. 1A). The brightness values and concentrations determined by MCF for the two components result in 33% binding (symbolized as dashed lines in Fig. 1B). MCF has been found to serve as a simple, fast, and robust analysis method to realize FIDA for screening applications (e.g., analysis time $\ll$ 1 s

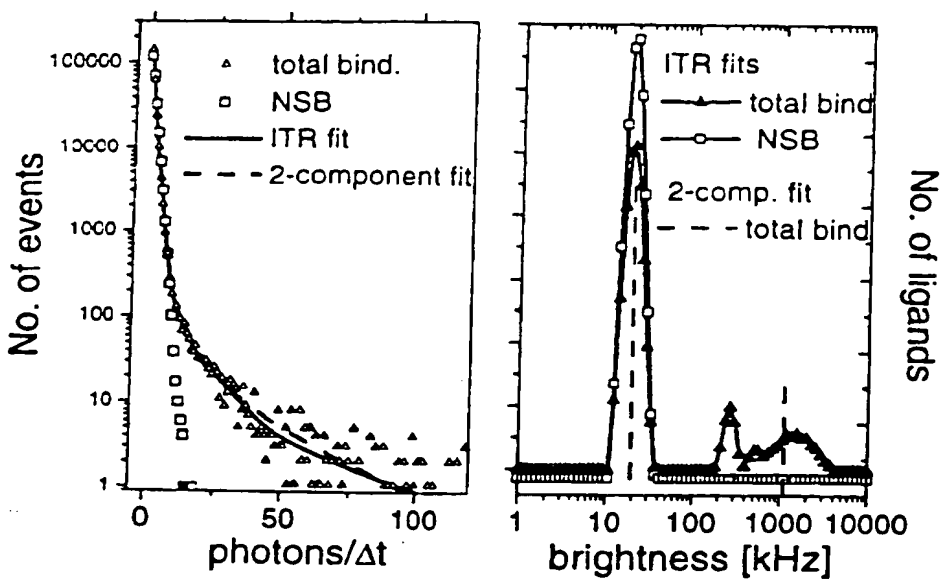

Figure 1. FIDA measurements and data analysis of TAMRA-MCH binding to MCH receptor membrane vesicles. (A) Photon counting histograms for total binding (1 nM TAMRA-MCH, 0.2 mg/ml membranes) and nonspecific binding (NSB) (an identical sample plus 1 μM unlabeled MCH). The curve representing total binding has been analyzed by ITR or MCF (allowing for two components). (B) The results of ITR analysis of the data in A can be visualized as brightness distribution curves showing the number of ligands versus specific brightness of the detected particles. The species found by MCF are symbolized by *dashed lines*. For clarity, the ITR curves are slightly offset along the y axis. For the total binding sample, ITR analysis gave 29% and MCF 33% binding.

per measurement with MCF, whereas ITR can take several seconds). All quantitative results discussed below have been obtained by MCF.

In FA measurements, ligand binding to membrane-bound receptors can be detected as a change in fluorophore molecular rotation rates.[6,12] However, if FA is measured on macroscopic detection systems such as the LJL plate readers,[8,20] only an average signal output from all fluorophores in the sample is monitored (i.e., the average fluorescence anisotropy weighted by concentrations and brightness values of the components). In contrast, a microscopic approach such as FIDA enables the direct and independent determination of the bound and free ligand concentrations in an assay mixture.[8,13] For samples as measured by FIDA in Figure 1, the corresponding anisotropy values are in the range of $r = 0.2$ (total-binding sample) or $r = 0.1$ (NSB sample) (e.g., Fig. 5D below). Hence, FA allows only indirect detection of ligand binding as an increase in overall anisotropy and requires a significant proportion of the ligand t be bound in order to generate a signal change, whereas FIDA can directly detect a small fraction of bound ligand and is therefore more sensitive and, presumably, robust.

FIDA binding assays for various GPCR subtypes

To illustrate the broad utility of the FIDA technique for measurement of GPCR ligand binding, we constructed assays for a range of receptors, representing all the major subtypes of GPCR–ligand pairings. Binding constants (K_d) and specific receptor densities (B_{max}) were determined by measuring dose–response curves for receptor binding while keeping either ligand or receptor concentration constant and varying the concentration of the other component. In all cases, specificity of binding has been confirmed by determination of apparent inhibition constants for displacement of the fluorescently labeled ligand with its unlabeled counterpart and/or low-molecular-weight antagonists. Representative data from these types of measurements are shown in Figure 2 for TAMRA MCH binding to the MCH receptor. A high signal and low unspecific binding (5%–10%) resulted in a large signal window. The standard deviations shown were determined from repeated readings of the same samples and therefore represent the variability of the detection method, excluding other sources of variability (e.g., pipetting errors). The separation between the signals corresponding to highest and lowest inhibitor concentrations in Figure 2C is characterized by a Z′ value[21] of 0.7, demonstrating that the precision of FIDA detection is HTS compatible.

Similar experiments are shown in Figure 3 for receptor–ligand pairs representing various ligand types: proteins (interleukin-8 [IL-8]; Fig. 3A), peptides (gastrin-releasing peptide [GRP]; Fig. 3B), and low-molecular-weight ligands (β_2-adrenergic receptor/CGP-12177; Fig. 3C). These examples also represent typical approaches to fluorescent labeling of ligands, which is a crucial step for configuring FIDA (or other fluorescence-based) binding assays. For the chemokine IL-8, a mutant was expressed with a single unpaired Cys within the C-terminal sequence, which was labeled with TAMRA-maleimide. For GRP, a truncated analog was designed (GRP-K9-27) in which a Rhodamine Green–labeled Lys was introduced at the N-terminus during solid-phase synthesis. The fluorescent ligand of the β_2-adrenergic receptor has been synthesized by attaching

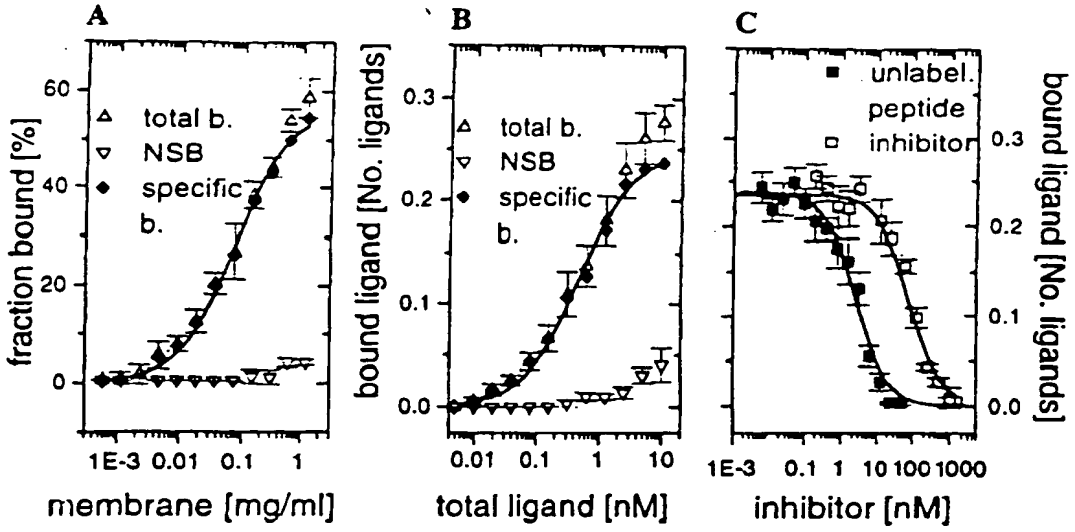

Figure 2. Binding and displacement of TAMRA-labeled MCH to MCH receptor membranes as measured by FIDA. (A) Membrane titration (at constant ligand concentration = 0.5 nM), resulting in K_d = 80 ± 8 μg/ml and B_{max} = 1.8 ± 0.05 pmol/mg. (B) Ligand titration (at constant membrane concentration = 80 μg/ml) gives K_d = 0.4 ± 0.05 nM. Binding is measured as the number of bound ligands detected in the confocal volume. (C) Displacement of TAMRA-MCH by unlabeled MCH or low-M_r antagonist (ligand concentration 1 nM, membrane concentration 0.1 mg/ml) resulting in IC_{50} values of 2.8 ± 0.3 nM and 100 ± 9 nM, respectively.

BODIPY FL via a linker to the antagonist CGP12177.[22] As determined by FIDA, the BODIPY-labeled compound showed subnanomolar potency similar to that of unlabeled CGP12177 (data not shown; see also ref. 22), and, when used in anisotropy assays, resulted in pIC_{50} values consistent with radioligand binding data.[12] The hydrophobic character of this ligand causes some accumulation in the membrane leading to a higher nonspecific background than observed for chemokine or peptide ligands (Fig. 3C), but a specific binding signal could still be obtained by FIDA. These examples underline the broad applicability of FIDA to various GPCR and ligand types.

To validate the suitability of FIDA for HTS and SAR screening, inhibition constants for a series of MCH antagonists were determined by FIDA, using displacement of TAMRA-MCH (Fig. 4A). The results were highly comparable to data from a standard radioligand filter binding (RLB) assay using iodinated

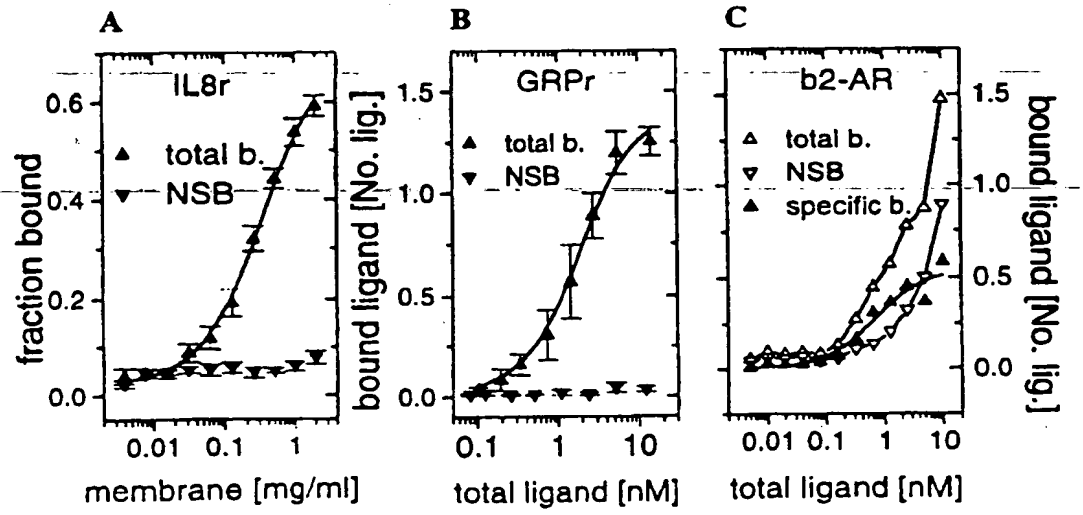

Figure 3. FIDA binding curves for a range of 7-transmembrane receptor and ligand types. (A) IL-8 chemokine receptor/TAMRA–IL-8 (K_d = 0.3 ± 0.03 mg/mL). (B) GRP receptor/Rhodamine Green-GRP (K_d = 1.2 ± 0.2 nM). (C) β_2-Adrenergic receptor/BODIPY FL-CGP12177 (K_d = 0.6 ± 0.2 nM).

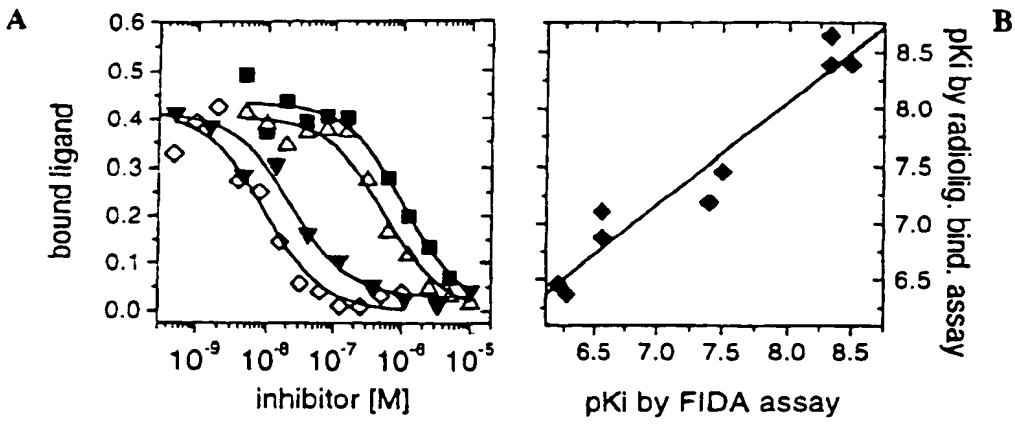

Figure 4. Comparison of binding competition data for a range of low-molecular-weight MCH antagonists between FIDA and radioligand binding. (A) Representative displacement curves for antagonists measured by FIDA. (B) Correlation between pK_i values obtained using either FIDA or radioligand filter binding assays. The three data points at the highest pK_i value represent unlabeled MCH.

MCH, with the average deviation between FIDA and RLB results within the range of day-to-day variability observed for either assay (Fig. 4B).

Sensitivity of FIDA and FA for 7-transmembrane receptor binding assays

FIDA and FA were applied to identical samples of MCH receptor membranes and TAMRA-labeled MCH. Titration of the membrane preparation at constant ligand concentrations resulted in comparable dose–response curves when measured by either of the methods (Fig. 5A and 5B). However, the data variability in the FIDA measurements were smaller, which allowed good assay statistics to be obtained at lower fractional ligand binding. Moreover, when the ligand was titrated at constant receptor concentration, only FIDA allowed quantification of the bound ligand and K_d determination (Fig. 5C), whereas the FA signal decreased at higher ligand concentration as fractional binding decreased. This reflects the fact that FIDA allows detection of concentrations of individual components, such as bound and unbound ligands, differing in their brightness values, whereas (macroscopic) FA only measures the overall average anisotropy of the mixture of fluors, as discussed above. Direct detection of the bound ligand by FIDA as a high-brightness component in the assay mixture is also crucial for suppressing interferences caused by compound autofluorescence, because compounds are usually much less bright. In addition, the confocal detection volume is less than 200 μm deep into the solution, minimizing the effects of absorbance of excitation and/or emission light by test compounds (i.e., inner-filter effects), and the effects of light scattering.

The stability of FIDA against autofluorescence and light scattering is a particular advantage compared with FA, wherein autofluorescence decreases the observed signal (because of the low degree of anisotropy from free low-molecular-weight compounds), whereas light scattering from precipitates of marginally soluble compounds generally increases observed anisotropy (because scattered light is fully polarized). These two factors, as the net result, usually increase variability from FA measurements in the presence of test compounds, particularly when operating close to the limits of this technique (i.e., low tracer concentrations or relatively small signal changes). Moreover, in FA measurements, light scattering contributions from membrane vesicles can also be significant (see below). This problem occurs in FIDA only to a much lesser extent.

Fractional binding can be determined from FIDA experiments by measuring the concentrations of both bound and unbound ligand. This can directly be compared to FA values measured on the same samples (Fig. 5D), indicating that the specific anisotropy change (i.e., the difference between the signals for total binding and NSB) is proportional to fractional binding. These data also illustrate the fact that FA can only be applied within a certain window of ligand concentrations (here around 0.5–1 nM at a given receptor concentration), because fractional binding decreases at higher concentrations. Therefore, FA assays for GPCR binding require highly sensitive plate readers. In addition to detector sensitivity, autofluorescence and light scattering from the membrane preparation define the limits of applicability for FA.

From these types of experiments, typical boundary conditions for statistically useful detection of specific binding (i.e., $Z' > 0$)[21] can be defined: fractional binding of 10% or greater for FIDA and 25% or greater for FA, and ligand concentration of 0.1 nM or greater for FIDA and 0.5 nM or greater for FA. For robust uHTS, a larger signal separation is required (e.g., $Z' > 0.4$), imposing further restrictions. In FA, the minimum ligand concentration is defined by background autofluorescence and light scattering caused by the membrane particles, and therefore largely depends on the specific density of receptors in the membrane. With a typical ligand concentration of 1 nM, a membrane concentration of maximally about 1 mg/ml (total protein concentration) can be tolerated, still yielding a sufficient signal:background ratio. Typical expression levels (e.g., from HEK293 cells) may yield 1 pmol receptor/mg membrane protein, meaning a maximal receptor concentration of 1 nM for an FA assay with 1 nM fluorescent ligand. The implications of these boundary conditions are plotted in Figure 6, assuming (for illustration) a receptor concentration of 1 nM (Fig. 6A), and

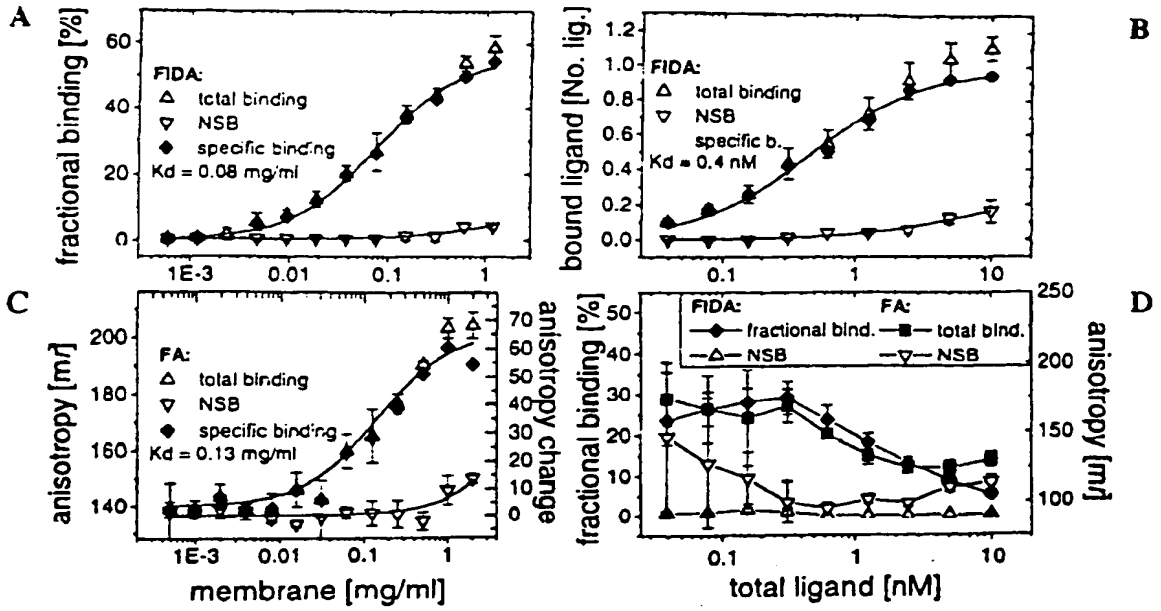

Figure 5. Comparison of data for binding of TAMRA-MCH to MCH receptor membranes using FIDA and FA. (A and B) Membrane titration (at constant ligand concentration = 0.5 nM) for FIDA (A) and FA (B). Specific binding in B is shown as anisotropy change (total binding − fitted NSB signal). (C and D) Ligand titration (at constant membrane concentration = 80 μg/ml) for FIDA (C) and FA (D). D also shows the relationship between the fractional binding determined by FIDA (from the same experiment as shown in C) with the overall anisotropy change observed in FA on the same samples.

K_d = 1 nM (Fig. 6B). A maximum of 75% binding was assumed because very high fractional binding reduces the sensitivity of assays to detect competitive antagonists (because, to a first approximation, $IC_{50} = K_i(1 + [R_t]/K_d)$).[6]

Two aspects of the increased working range of FIDA for fluorescence-based GPCR binding assays are worth noting. First, high K_d values (e.g., Fig 6A) may be encountered, particularly if attachment of fluorescent labels decreases the affinity of the natural ligand. Second, the ability to work at lower receptor concentrations (e.g., Fig. 6B) allows the use of cell lines with lower receptor expression (potentially even primary cells), or lower concentrations of membranes in order to conserve biological reagents. The ability to measure low fractional binding by FIDA may be a crucial advantage for assay configuration, particularly because cell lines engineered for measuring functional responses (e.g., to identify orphan receptor–ligand pair-

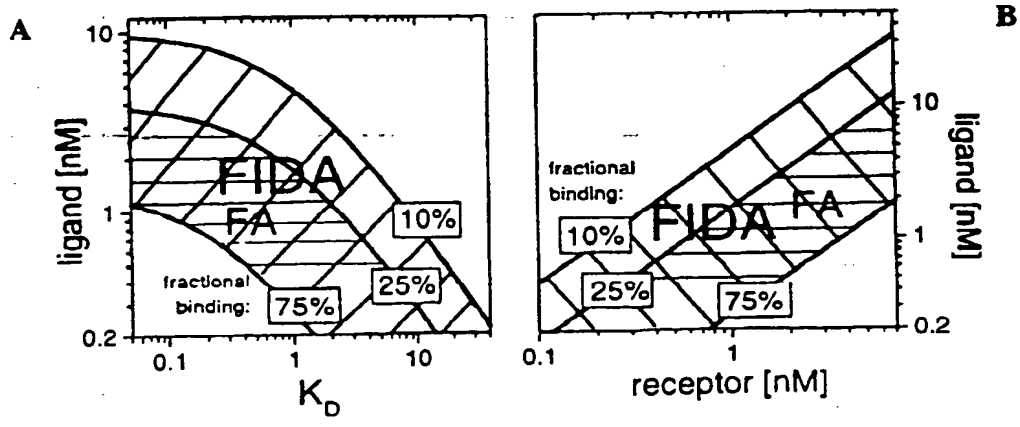

Figure 6. Examples of calculated boundary conditions for FIDA and FA assays for 7-transmembrane receptor ligand binding est... mated from experiments similar to that shown in Figure 5. Contour lines of constant fractional binding were calculated for a recep... concentration of 1 nM (A) or K_d = 1 nM (B). The range where FA is applicable is indicated by horizontal hatch; FIDA is appli... to a broader range of conditions.

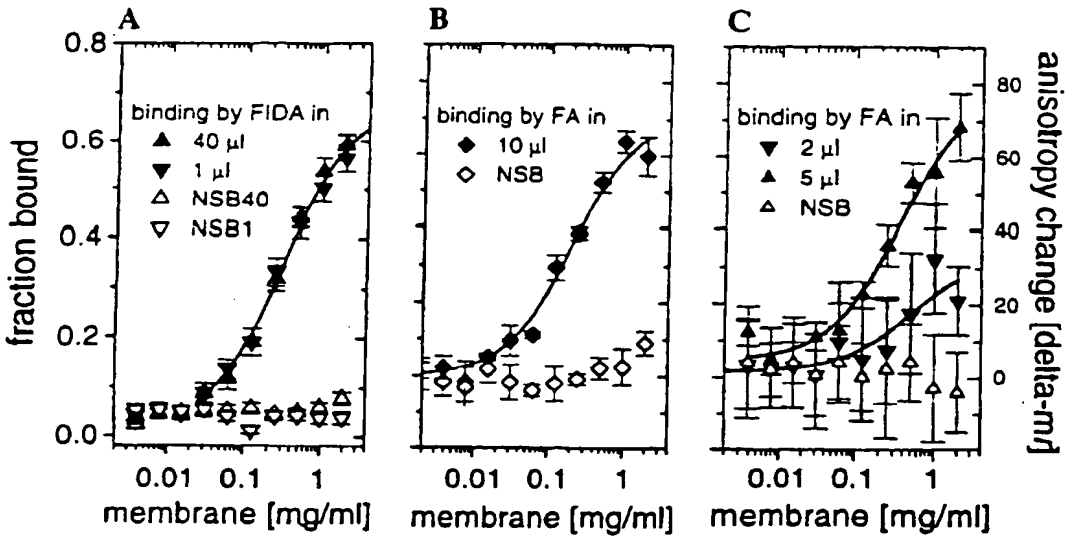

Figure 7. Miniaturization of FIDA and FA assays for 7-transmembrane ligand binding. (A) Binding curve for the IL-8 receptor using TAMRA-IL-8 measured by FIDA. Miniaturization of the assay volume from 40 to 1 µl (taken from the same sample) has no effect on binding data. (B) Typical FA data in an assay volume of 10 µl (1536-well plate), here representing TAMRA-MCH binding to MCH receptor membranes, showing slightly higher variability but a sufficient signal separation for screening. (C) The same samples as measured by FIDA in A here measured by FA in assay volumes of 5 or 2 µl (LJL-HE 384 plates) showing that the signal is degrading as the volume is lowered. The FA data are plotted as anisotropy change relative to the anisotropy of free ligand

ings) do not always show sufficiently high expression as required for FA or other formats of binding assays. In summary, FIDA allows a wider working range, while FA is useful within the more restricted boundary conditions of high-affinity interaction and high expression level.

Miniaturization and uHTS capability

In FIDA, fluorescence is detected in a confocal volume element of about 1 fl, independent of the total assay volume. Therefore, the FIDA signal is insensitive to miniaturization down to very low assay volumes.[6–8] This is demonstrated in Figure 7A, which shows binding curves for TAMRA–IL-8/IL-8–receptor measured in either 40- or 1-µl sample volume yielding identical results. The LJL Acquest plate reader (which uses semiconfocal optics) is capable of measuring FA signals in volumes in the microliter range at nanomolar concentrations. As an example, binding of TAMRA-MCH to MCH receptor membranes was measured on the LJL Acquest in an assay volume of 10 µl (Fig. 7B). The same samples as analyzed by FIDA (Fig. 7A) were also measured using FA in a 5- or 2-µl sample volume (Fig. 7C). The FA signal degraded when the assay volume was reduced, demonstrating the limit of macroscopic detection used for FA measurements (as opposed to microscopic detection for the confocal FIDA readout).[8]

FA measurements can be performed with short integration times (e.g., 0.1 s) per well, allowing uHTS-compatible sample throughput (e.g., 100,000 wells/day). All FIDA data shown here were measured with 10 s read time/well. The read time for FIDA can be decreased to 2–4 s (depending on the particular receptor–ligand system), which allows for a throughput of 20,000–40,000 wells/24 h and per reader channel. Further increase of the throughput to meet uHTS demands will be achieved by parallelization of the confocal reader. Shorter read times can also be achieved by employing two-color techniques where both binding partners (ligand and receptor or ligand and vesicle) are fluorescently labeled.[8,23,24] Therefore, uHTS-compatible throughput can be achieved by confocal detection methods with the additional benefit of intrinsic miniaturizability and high information content as described above.

CONCLUSIONS

Homogeneous fluorescence-based assay formats for GPCR ligand binding have been demonstrated that are applicable to all ligand/receptor classes and that have characteristics well suited to miniaturized uHTS applications. FIDA, based on confocal detection of fluorescence fluctuations, has marked advantages over the more conventional approach of FA, measured on a plate reader with macroscopic detection optics, particularly with respect to the requirements for receptor concentration and K_d. In fact, FIDA appears to work best at modest receptor expression levels because of the need to detect free and bound ligand within the instrumental dynamic range. In cases where receptor expression is not limiting, we have shown that FA can be a useful technique, although requiring larger assay volumes. In addition, the definition of boundary conditions for either technique with respect to K_d, receptor and ligand concentration, and assay volume as shown here can improve predictability of assay design and lower cycle times by establishing quantitative criteria for successful configuration of fluorescence-based GPCR ligand binding assays.

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REFERENCES

1. Iismaa TP, Biden TJ, Shine J: *G Protein-Coupled Receptors: Molecular Biology Intelligence Unit.* Heidelberg: Springer Verlag. 1995.
2. Stadel JM, Wilson S, Bergsma DJ: Orphan G protein-coupled receptors: a neglected opportunity for pioneer drug discovery. *Trends Pharmacol Sci* 1997;18:430–437.
3. Schroeder KS, Neagle BD: FLIPR: a new instrument for accurate, high throughput optical screening. *J Biomol Screen* 1996;1:75–80.
4. Jurewicz AJ, Foley JJ, Stewart R, et al: Fluorometric imaging plate reader: using the orexin receptor to screen 145,000 compounds. *Genet Eng News* 1999;19:44–46.
5. Roemer K, Tsien RY: Quantitation of transcription and clonal selection of single living cells with beta-lactamase as reporter. *Science* 1998;279:84–88.
6. Pope AJ, Haupts UM, Moore KJ: Homogeneous fluorescence readouts for miniaturised high-throughput screening: theory and practice. *Drug Discov Today* 1999;4:350–362.
7. Moore KJ, Turconi S, Ashman S, et al: Single molecule detection technologies in high throughput screening: fluorescence correlation spectroscopy. *J Biomol Screen* 1999;1:335–353.
8. Haupts U, Rüdiger M, Pope AJ: Macroscopic versus microscopic fluorescence techniques in (ultra)-high-throughput screening. *Drug Discov Today HTS Suppl* 2000;1:3–9.
9. Cook ND: Scintillation proximity assay—a versatile high throughput screening technology. *Drug Discov Today* 1996;1:287–294.
10. Mellentin-Michelotti J, Evangelista LT, Swartzman EE, et al: Determination of ligand binding affinities for endogenous seven-transmembrane receptors using fluorometric microvolume assay technology. *Anal Biochem* 1999;272:182–190.
11. Zuck P, Lao ZG, Skwish S, et al: Ligand-receptor binding measured by laser-scanning imaging. *Proc Natl Acad Sci U S A* 1999; 96:11122–11127.
12. Allen M, Reeves J, Mellor G: High throughput fluorescence polarization: a homogeneous alternative to radioligand binding for cell surface receptors. *J Biomol Screen* 2000;5:63–69.
13. Kaask P, Palo K, Ullmann D, et al: Fluorescence-intensity distribution analysis and its application in biomolecular detection technology. *Proc Natl Acad Sci U S A* 1999;96:13756–13751.
14. Evotec Biosystems AG: International Patent Application PCT/EP97/05619 and International Publication No. WO 98/16814, 1998.
15. Auer M, Moore KJ, Meyer-Almes FJ, et al: Fluorescence correlation spectroscopy—lead discovery by miniaturised HTS. *Drug Discov Today* 1998;3:457–465.
16. Fries JR, Brand L, Eggeling C, et al.: Quantitative identification of different single molecules by selective time-resolved confocal fluorescence spectroscopy. *J Phys Chem A* 1998;102:6601–6613.
17. Chen Y, Muller JD, Seo PTC, et al: The photon counting histogram in fluorescence fluctuation spectroscopy. *Biophys J* 1999; 77:553–567.
18. Chambers J, Ames RS, Bergsma D, et al: Melanin-concentrating hormone is the cognate ligand for the orphan G-protein-coupled receptor SLC-1. *Nature* 1999;400:261–265.
19. Biemond J, Lagendijk RL, Mersereau RM: Iterative methods for image deblurring. *Proc IEEE* 1990;78:856–883.
20. Sportsman JR, Leytes L: Miniaturization of homogeneous assays using fluorescence polarization. *Drug Discov Today HTS Suppl* 2000;1:27–32.
21. Zhang JH, Chung TDY, Oldenburg KR: A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* 1999;4:67–73.
22. Heithier H, Hallmann D, Doege F, et al: Synthesis and properties of fluorescent beta-adrenoceptor ligands. *Biochemistry* 1994;33: 9126–9134.
23. Schwille P, Meyer-Almes FJ, Rigler R: Dual-colour fluorescence cross-correlation spectroscopy for multicomponent diffusional analysis in solution. *Biophys J* 1997;72:1878–1886.
24. Winkler T, Kettling U, Koltermann A, et al: Confocal fluorescence coincidence analysis: an approach to ultra high-throughput screening. *Proc Natl Acad Sci U S A* 1999;96:1375–1378.
25. Scheel AA, Funsch B, Bosch M, et al: Receptor-ligand interactions studied with homogenous fluorescence-based assays suitable for miniaturized screening. *J Biomol Screen* 2001;6:11–18.

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